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14. ABSTRACT
The5-10% of NF1 patients who harbor so-called microdeletions （μΔ) often develop high numbers of neurofibromas at an early age. Similar enhanced tumorigenesis is not seen in pateinets with smaller deletions that remove just the NF1 gene, arguing that one or more of the ~ 20 μΔ genes acts as a tumor burden modifier. Underlying this project was our finding that μΔ fibroblast and Schwann cells over-express multiple E2F/DP target genes, and that siRNA mediated suppression of C17orf79/COPR5 partially phenocopied this expression signature. However, results obtainted to date have cast doubt on our hypothesis C17orf79/COPR5 is the long sought for NF1 μΔ tumor burden modifier. Thus, new μΔ containing fibroblasts failed to show the E2F/DP target gene over-expression signature, and while it is possible that trivial reasons are responsible for this negatie result, work by others has implicated another μΔ gene, SUZ-12 as a likely tumor burgen modifier. Whether C17orf79/COPR5 acts in conjunction with SUZ12 to promote tumorigenesis remains to be determined.

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Table of Contents

Introduction

The approximately 5-10% of NF1 patients who harbor 1.2-1.4 megabase *NF1* gene centered microdeletions (μΔ) tend to develop unusually high numbers of neurofibromas at an early age. Similar enhanced and/or accelerated tumorigenesis is not seen in patients with smaller deletions that remove just the *NF1* gene, arguing that at least one of the roughly 20 genes that are co-deleted in patients with *NF1* μΔ syndrome acts as a tumor burden modifier. The long-term goal of all our NF1-related research has been the identification of NF1 modifier genes, which control rate limiting steps during disease development and as such represent pre-validated therapeutic targets. To identify the NF1 μΔ tumor burden modifier, work supported by this idea/hypothesis development award was based on our finding that μΔ containing fibroblasts and Schwann cells consistently over-express broad sets of cell proliferation associated genes controlled by E2F/DP transcription factors, compared to similar non-μΔ cells. Our preliminary data had implicated the C17orf79/*COPR5* gene (hereafter referred to by its current official *COPRS* gene name) as a likely contributor to the aberrant gene expression, and our main objective was to provide further evidence that *COPRS* may be the long sought for NF1 μΔ tumor burden modifier.

Body

Multiple cell proliferation associated genes controlled by the E2F/DP family of heterodimeric transcription factors show highly significant, approximately 2-fold increased expression in μΔ compared to non-μΔ fibroblast and Schwann cells. This robust expression signature suggests that μΔ cells are in a more proliferative state. Two main findings implicated μΔ gene *SUZ12* as a likely candidate tumor burden modifier. First, Dr. Eric Legius, who had provided us with the fibroblast and Schwann cell RNAs for our study, had identified *SUZ12* mutations in two cases of malignant peripheral nerve sheath tumor. Secondly, *SUZ12* encodes a subunit of Polycomb Repressive Complex 2 (PRC2) involved in the epigenetic regulation of gene expression, and another PRC2 subunit, *EZH2*, is frequently mutated in various forms of cancer [1]. Thus, our initial hypothesis was that reduced *SUZ12* expression might be responsible for the aberrant gene expression signature and for enhanced tumorigenesis. To begin to test this hypothesis we analyzed whether a reduction *SUZ12* expression phenocopied the E2F/DP target gene over-expression signature. However, in work supported by an earlier Idea/Hypothesis Development Award, we found that reducing *SUZ12* expression in glial and other cell types resulted in high levels of cell death and, in surviving cells, strongly reduced rather than increased expression of E2F/DP controlled genes. This presumably reflects the fact that in many cell types *SUZ12* is an essential gene [2].

During the course of this earlier project it was reported that the product of another μΔ gene, *COPRS*, also serves as an epigenetic regulator, as the targeting subunit of the PRMT5 protein arginine methyl transferase. Specifically, it was shown that COPRS interacted with PRMT5, and that both proteins were novel subunits of a Cyclin E Repressive Complex (CERC), which also includes E2F4, DP1, and members of the retinoblastoma family. Dissociation of CERC from an atypical E2F binding site in a so-called Cyclin E Repressor Module (CERM) within the *CCNE1* promoter allowed the transient induction of this canonical E2F/DP target gene at the G1/S boundary. Mechanistically, COPRS was shown to alter the substrate specificity of PRMT5 towards Histone H4 arginine 3 (H4R3), creating the H4R3-dimethyl repressive epigenetic mark within CERM, but not in adjacent DNA [3]. Reduced *CORPS* expression in microdeletion cells might therefore increase the expression of *CCNE1* and perhaps other E2F/DP target genes, and our preliminary data showed that siRNA-mediated suppression of indeed increased the expression of *CCNE1* and *MCM6*, another canonical E2F/DP target gene. We proposed two

mechanisms whereby a reduction in *COPRS* expression might induce the expression of E2F/DP target genes. The first involved the de-repression of the critical cell cycle regulator *CCNE1* and perhaps other E2F/DP regulated genes upon reductions in PRMT5/COPRS-mediated H4R3 dimethylation. The second mechanism was based on the finding that PRMT5 can directly methylate E2F1, leading to its stabilization [4]. Although COPRS had not been implicated in this second pathway, we speculated that a reduction in PRMT5/COPRS complex formation might increase the ability of PRMT5 to stabilize E2F1. Based on these findings the three specific aims of the current project were:

1. To test whether short or longer term *COPR5/PRMT5* KD phenocopies the E2F/DP expression signature of μΔ fibroblasts.
2. To test whether μΔ and non-μΔ fibroblasts differ in the level of H4R3 methylation at the promoters of COPR5 regulated genes, or in PRMT5-controlled E2F1 stability.
3. To test whether COPR5 re-expression normalizes E2F/DP target gene expression in μΔ fibroblasts.

Before describing progress towards achieving these aims, let me explain the reasons behind the current 6-month no-cost extension. First, this project started approximately 6 month after the proposed start date due to the sequestration of government funding. This delay adversely affected the project in two ways. First, we had proposed using relatively low cost multiplexed 135k NimbleGen human expression arrays to analyze expression profiles in several of our experiments. However, by the time the project received funding, NimbleGen had abandoned the array business, forcing us to find an alternative cost-effective method. Among the few available alternatives, we chose Agilent 8-fold multiplex arrays as a reasonable substitute. Although the Agilent people argued that their technology should be up and running in 2-3 months, in reality it took almost 9 month before the first analysis was completed. This of course badly affected the time-line of this one-year project. A second problem related to the delayed start date was that the highly experienced technician who was going to devote 100% effort towards this project became discouraged and left my lab. Thus, another person had to be found and trained in biosafety procedures required for lentiviral work and various other specialized procedures, which caused further delays.

In our Statement of Work we had proposed the following sub-aims and time lines to achieve our three specific aims. Each sub-aim in italic text is followed by comments in plain text describing progress achieved to date.

Tasks to achieve Aim 1:

Month 1-3 *Test level of knockdown (KD) achievable with 6 available COPR5 and 7 available PRMT5 shRNA lentiviral vectors.*

This task was successfully completed. Briefly, we generated 6 COPRS and 7 PRMT5 lentiviral stocks, infected IMR90 human fibroblasts, applied drug selection, and quantified target gene knockdown at various times post infection. In the case of PRMT5, knockdown was verified by immunoblot analysis using a commercially available antibody. No such antibody was available for COPRS, so in this case we analyzed gene knockdown by semi-quantitative RT-PCR analysis. Going beyond our original proposal, we also tested a non-commercial COPRS antibody provided by Dr. Eric Fabbrizio [3]. However, using cells transfected with a HA-tagged COPRS as a positive control, the affinity-purified rabbit antibody did not reliably detect either endogenous or ectopically expressed COPRS protein.

Using blots and RT-PCR to assess the efficacy of *PRMT5* and *COPRS* knockdown, we found that only two *PRMT5* targeting viruses failed to show any appreciable knockdown after 48 hrs. The 11 other viruses all produced various levels of *PRMT5* or *COPRS* knockdown, ranging from 35-95%. We also tested the level of knockdown using the same viruses 24 hrs after infection, but found knockdown to be much less effective at this earlier time point.

Month 1-6 *Perform short-term siRNA-mediated and longer-term shRNA mediated COPR5 KD using IMR90 fibroblasts. Perform long-term shRNA-mediated PRMT5 KD. Prepare RNA from KD and control cells and perform NimbleGen expression profiling using 12-plex arrays. Identify genes co-regulated by COPR5 and PRMT5, and evolutionary conserved PRMT5 KD targets by comparing obtained expression profiles with each other, and with published murine Prmt5 KD profiles.*

Transfecting cells with Dharmacon Smartpool siRNAs successfully achieved short-term (24 hr) COPRS knockdown. As explained above, it has taken 9 months to make the switch from NimbleGen to Agilent array technology. While the array hybridization of RNAs from three biological COPRS, PRMT5 and scrambled control 48 hr knockdown replicates has been completed, the longer-term knockdown and overall data analysis remain incomplete. All that can be said at this time is that we have seen changes in gene expression upon 48 hr *PRMT5* or *COPRS* knockdown, but how reproducible these changes are, in what way they relate to the altered expression profiles of μΔ cells, or to what extent human *PRMT5* target genes overlap with previously reported murine *Prmt5* targets [5], are all issues that remain to be determined.

Month 4-6 *To allow straightforward comparison of results, use NimbleGen arrays to similarly compare expression profiles of **μΔ** and non-**μΔ** fibroblasts.*

We had proposed to re-profile two μΔ and three non-μΔ fibroblasts on the new array platform to allow straightforward comparison with previous results. Unfortunately, the old RNAs didn't pass the standard quality control test required before any array hybridization. However, bioinformatics specialists convinced us that comparisons between array platforms are routine, and that the proposed re-profiling wasn't really necessary. Rather, to gain experience with the new array platform we decided to instead analyze gene expression profiles of the new μΔ and non-μΔ fibroblast lines provided by Dr. Legius. Because in previous work the E2F/DP target gene over-expression was consistently observed in fibroblasts and Schwann cells, our expectation was that we would have no trouble reproducing our original result. However, Agilent array analysis of three μΔ and two non-μΔ fibroblast lines grown in our lab (a third non-μΔ fibroblast line did not grow) failed to reveal any obvious expression differences. Thus, unsupervised hierarchical clustering did not separate μΔ from non-μΔ cells, and Gene Set Enrichment Analysis (GSEA) detected no significant overlap with any previously defined E2F/DP regulated or other functional gene sets. These results are in stark contrast to previous data, when unsupervised clustering clearly distinguished μΔ and non-μΔ cells, and when GSEA found highly significant overlap with multiple E2F/DP controlled gene sets.

At least two factors may explain this unexpected negative result. The first is that we previously analyzed fibroblast and Schwann cell RNAs provided to us by Dr. Legius. For the current project, Dr. Legius provided frozen stocks of tumor-derived fibroblasts to grow in our own lab. This should have been straightforward, but for reasons that remain hard to fathom the cells grew poorly, and died after only few passages. Thus, the fact that the three μΔ fibroblasts lines did not

show elevated E2F/DP target gene expression might simply reflect the fact that the cells at the time of RNA extraction were unhealthy. A different explanation that we cannot entirely dismiss is that the cells provided were not really μΔ cells. Thus, in previous work we did observe a clear-cut approximately 50% reduced expression of several μΔ genes in μΔ cell derived RNAs. In this case, we did not see any difference in the expression of the same μΔ genes between the two groups of cells.

In our proposal we had not anticipated that the different gene expression profile of μΔ cells might not be reproducible, or that the cells provided to us would be difficult to grow. In fact, growing tumor-derived fibroblasts should be a routine procedure. If we had encountered these unexpected results earlier in this one-year project, we could have requested replacement cells, or made alternative arrangements. Unfortunately, this is not possible at this late stage.

Month 6-12 *Result analysis, repeat and follow-up experiments as needed.*

Analysis of gene profiling results required us to familiarize ourselves with novel proprietary software and remains incomplete. No repeat experiments are anticipated.

Tasks to achieve Aim 2:

Month 1-4 *Establish and gain experience with μΔ and non-μΔ fibroblast lines provided by Dr. Legius. Perform test runs of ChIP/PCR and ChIP/Seq experiments, determining suitable experimental conditions.*

Months 2-9 *Perform ChIP/PCR experiments with IMR90 lines showing different levels of stable COPR5 KD to analyze changes in CCNE1 promoter CERM element H3R4 dimethylation. Similarly analyze differences in H3R4 dimethylation levels at the promoters of other genes co-regulated by COPR5 and PRMT5 KD. Perform ChIP/Seq analysis to assess genome-wide changes in H3R4 dimethylation between μΔ and non-μΔ fibroblasts*

As described above, we have been unable to grow the μΔ and non-μΔ fibroblast lines beyond a limited number of passages. Whether this was due to the relative inexperience of the new person hired for this project, to delays in U.S. Customs clearance of the frozen cells, or to other factors, is impossible to decide at this time. However, whatever the reason, our inability to grow these cells made it impossible to perform several proposed experiments, including the ChIP/PCR and ChIP/Seq experiments to achieve the first part of this second aim. Before this problem became apparent, we did use parent and COPR5 knockdown IMR90 fibroblasts to optimize chromatin isolation and cross-linking procedures in preparation for ChIP/PCR and ChIP/Seq assays. However, even though these procedures were successfully established, the critical experiments to assess gene-specific and genome-wide H3R4 dimethylation levels in μΔ and non-μΔ fibroblasts have been impossible to perform.

Months 6-9 *Test whether PRMT5 knockdown and over-expression reproduces reported effect on E2F1 stability. Compare E2F1 protein level and stability between μΔ and non-μΔ fibroblasts.*

Again, our inability to maintain long-term cultures of μΔ and non-μΔ fibroblasts interfered with our plan to directly compare E2F1 protein levels in these cells. However, we did analyze E2F1 protein levels in IMR90 cells in which *PRMT5* or *COPRS* had been knocked down by lentiviral

shRNA expression. In replicate experiments that analyzed cells subjected to different levels of knockdown, we detected no obvious differences in E2F1 protein level. Others previously reported that depletion of *PRMT5* was associated with a 5-10-fold increase in E2F1 protein abundance in human U2OS cells (4). Experiments to determine why our results in IMR90 cells differ from those reported in U2OS cells remain incomplete at this time.

Months 8-12 *If increased E2F/DP activity not attributable to altered arginine dimethylation is found, analyze other components of CDK4/6/E2F/DP/RB signaling pathway.*

These experiments again required healthy μΔ and non-μΔ fibroblast cells. In their absence, this proposed follow-up analysis has not been possible.

Month 8-12 *Result analysis, repeat and follow-up experiments as needed.*

We are conducting final experiments to further analyze the effects of *PRMT5* and *COPRS* depletion on E2F1 protein stability.

Tasks to achieve Aim 3:

Months 1-3 *Generate and characterize lentiviral epitope-tagged COPR5 expression vector.*

After two unsuccessful attempts that produced viruses that failed to direct detectable epitope-tagged *COPRS* expression, a functional N-terminally HA-tagged lentiviral *COPRS* expression vector was generated. Infection of IMR90 cells with this vector does lead to the production of the expected HA-tagged COPRS protein, as analyzed by immunoblot analysis.

Months 4-8 *Determine whether COPR5 re-expression normalizes the E2F/DP expression signature of μΔ fibroblasts.*

Our inability to grow the required μΔ fibroblasts made it impossible to perform this part of the project. The fact that the previously detected E2F/DP target gene over-expression signature was absent in short-term cultures of these cells, also removed its rationale.

Month 8-12 *Result analysis, repeat and follow-up experiments as needed.*

Key Research Accomplishments

On the positive side, we did successfully establish procedures that allow both short-term and longer term *COPRS* and *PRMT5* knockdown. We also successfully transitioned to the Agilent gene expression analysis platform, established critical procedures for ChIP/PCR and ChIP/Seq experiments, and generated an epitope-tagged lentiviral COPRS expression vector. However, although all preliminary steps for the proposed project were successfully completed, our inability to grow the μΔ and non-μΔ fibroblasts required for many experiments has been a serious obstacle standing in the way of successfully completing this project within the proposed one-year funding period.

Reportable Outcomes

If our ongoing analysis shows that *COPRS* knockdown produces a reasonable phenocopy of our previously detected E2F/DP target gene over-expression signature, we will publish this result so others can continue work to further implicate *COPRS* in NF1 tumor development.

Conclusions

We had not anticipated that the previously detected robust E2F/DP target gene over-expression signature would be absent in additional μΔ fibroblasts. Although this unexpected result cast doubt on the hypothesis underlying this proposal, it remains possible that poor cell health or other technical reasons are responsible, and that our original observations were correct. We are leaning towards this interpretation, because our preliminary data showed that *COPRS* knockdown produces at least a partial phenocopy of the μΔ fibroblast and Schwann cell expression signature. However, although we previously found that reducing *SUZ12* expression suppressed rather than enhanced the expression of E2F/DP regulated genes, two as yet unpublished findings by others strongly suggest a role for *SUZ12* as a NF1 tumor burden modifier. Thus, the Legius lab has detected *SUZ12* mutations in a substantial proportion in NF1 associated malignant peripheral nerve sheath tumors. Moreover, the Cichowski lab has found that combined genetic loss of murine *Nf1* and *Suz12* significantly enhances tumorigenesis.

We conclude, therefore, that although it remains possible that heterozygous loss of *COPRS* contributes to tumorigenesis by increasing the expression of multiple proliferation-associated genes, loss of *SUZ12* also likely plays a role. Whether *COPRS* and *SUZ12* both contribute to enhanced tumorigenesis, or which of these two genes is more important, are issues that remain unresolved and that will have to be addressed in future work.

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Appendices

None